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14. ABSTRACT
This grant investigates the potential use of calcitriol regulation of mullerian inhibitory substance (MIS) expression as an incremental therapy for prostate cancer. We have established that calcitriol (1,25-dihydroxyvitamin D3) directly stimulates MIS expression by binding to the vitamin D receptor (VDR) and directing the hormone-receptor complex to bind to a vitamin D regulatory element (VDRE) in the MIS promoter. We have attempted to demonstrate that combination of calcitriol and MIS achieve increased potency to inhibit prostate cancer cell growth compared to either drug alone. However, this has not yet been accomplished. We further demonstrated the interaction of calcitriol-VDR with other transcription factors, SF-1, SOX-9and GATA-4, to act together to synergistically increase MIS expression in prostate cancer cells. We believe that some of calcitriol's action to inhibit prostate cancer cell growth is due to stimulation of MIS and this work plans to substantiate this hypothesis and lay the ground work to translate this information to clinical trials in men with prostate cancer.

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INTRODUCTION

We propose that MIS regulation may be an important element contributing to the ability of 1,25-dihydroxyvitamin D_3 (calcitriol) to inhibit the growth and progression of prostate cancer cells. We have shown that calcitriol acts by several pathways to inhibit the growth of prostate cancer (PCa) cells [1-6]. In recent studies we showed that calcitriol, in addition to multiple other pathways, also stimulates the expression of MIS in a classical human prostate cancer cell line, LNCaP. We further show that the up-regulation of MIS expression is mediated directly by calcitriol binding to the vitamin D receptor (VDR) and the hormone-receptor complex subsequently interacts with a vitamin D regulatory element (VDRE) in the MIS gene promoter. In addition we have begun investigating the potential role of MIS in the diseases polycystic ovarian syndrome (PCOS). This is to consider whether to pursue future studies of calcitriol in ovarian cancer And we have also started very preliminary studies to combine MIS protein with calcitriol to ascertain whether the combination is more powerful to inhibit PCa cell growth than either drug alone.

BODY

1. Use of MIS plus calcitriol in combination to treat prostate cancer

One goal of the grant was to determine whether the effects of the combination of calcitriol and MIS on prostate cancer growth were better than either drug alone. MIS is a member of the transforming growth factor-beta (TGF□) superfamily of secreted protein hormones that signal through receptor complexes of type I and type II serine/threonine kinase

receptors. We have obtained purified MIS from Dr. David MacLaughlin at Harvard Medical School. In our first experiment, we treated LNCaP prostate cancer cells with calcitriol alone, MIS alone and the combination of calcitriol and MIS. As shown in Fig. 1, when cells were treated with calcitriol alone the growth of the LNCaP cells was inhibited in a dose-dependent manner. At the 10 nM calcitriol concentration growth was inhibited by more than 50%. In contrast, cells treated with MIS alone were not growth inhibited. The combination of calcitriol and MIS also caused growth inhibition but was only as effective as calcitriol alone. These results suggested to us that the MIS preparation was inactive or that the MIS type II receptor (MISRII) protein or the type I receptor ALK2 protein involved in MIS signal transduction pathway are not expressed in the LNCaP cells that we used. We plan to examine the LNCaP cells for MISRII and ALK2 expression. We have recently received a new batch of MIS and will repeat the experiments.

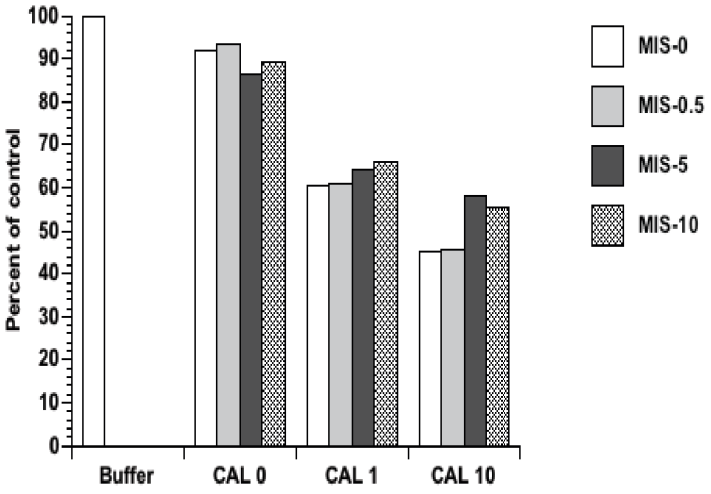

Fig. 1. Effects of calcitriol, MIS and the combination of calcitriol and MIS on LNCaP prostate cancer cell growth. Cells were grown in 6-well plates and treated with ethanol (CAL 0), 1 nM calcitriol (CAL 1) and 10 nM calcitriol (CAL 10) in the absence (MIS-0) and presence of MIS at 0.5 ug/ml (MIS-0.5), 5 ug/ml (MIS-5), and 10ug/ml (MIS-10). Cells were treated every other day for 6 days. Cell growth was determined by measuring DNA content. Values represent percent of buffer control set at 100%.

2. Other regulators of MIS expression

We have also been investigating the role VDR and of other transcription factors that regulate MIS gene expression. We have previously shown that steroidogenic factor 1 (SF-1) upregulates MIS gene expression. When we coexpressed the VDR with SF-1 there was a substantial increase in gene expression when the cells were treated with calcitriol. The transcription factors GATA-4 and SOX9 have also been shown to regulate MIS gene expression. We have obtained a GATA-4 expression vector from Dr. Mona Nemer and a SOX9 expression vector from Dr. Benoit deCrombrugghe. In this set of experiments, we transfected HeLa cells with the various transcription factors and the MIS promoter-luciferase construct. We then treated the cells with graded concentrations of calcitriol and determined luciferase activity. As shown in Fig. 2, cells transfected with VDR alone exhibited a dose-dependent increase in MIS promoter activity reaching a maximum of 1.8 fold in these experiments. When SF-1 was cotransfected with the VDR there was a 2-fold increase in promoter activity in the absence of calcitriol (vehicle treated sample). When calcitriol was added there was a dose-dependent increase in MIS promoter activity reaching a maximum 2-fold increase at the 100 nM calcitriol concentration. When SOX9 was cotransfected with the VDR there was no increase in promoter activity in the absence of calcitriol (vehicle treated sample). When calcitriol was added there was a dose-dependent increase in MIS promoter activity reaching a maximum 2-fold increase at the 100 nM calcitriol concentration. These results are similar to cells transfected with VDR alone suggesting that SOX9 does not enhance VDR actions to stimulate MIS promoter activity. When GATA-4 was cotransfected with the VDR there was also no increase in promoter activity in the absence of calcitriol (vehicle treated sample). When calcitriol was added there was a dose-dependent

increase in MIS promoter activity reaching a maximum 2-fold increase at the 100 nM calcitriol concentration. These results suggest that GATA-4 modestly enhances VDR actions to stimulate MIS promoter activity. We then cotransfected cells with VDR, SF-1, and SOX9. In the absence of calcitriol, the promoter activity was reduced compared to promoter activity in cells transfected with VDR and SF-1. However, addition of calcitriol resulted in a dose-dependent increase in promoter activity achieving a maximum 3-fold increase at the 100 nM calcitriol concentration. We then cotransfected cells with VDR, SF-1, and GATA-4. In the absence of calcitriol, the promoter activity was increased compared to the promoter activity in cells transfected with VDR and SF-1. Addition of calcitriol resulted in a dose-dependent increase in promoter activity achieving a maximum 1.6-fold increase at the 100 nM calcitriol concentration. We then cotransfected cells with VDR, SF-1, SOX9, and GATA-4. In the absence of calcitriol, the promoter activity was increased compared to the promoter activity in cells transfected with VDR and SF-1. Calcitriol treatment resulted in a dose-dependent increase in promoter activity achieving a maximum 1.8-fold increase at the 100 nM calcitriol concentration. These results demonstrate that the transcription factor GATA-4 enhances VDR actions on the MIS promoter. Together with VDR and SF-1, GATA-4 further increases MIS promoter activity. SOX9 also enhanced VDR actions but only when SF-1 was present.

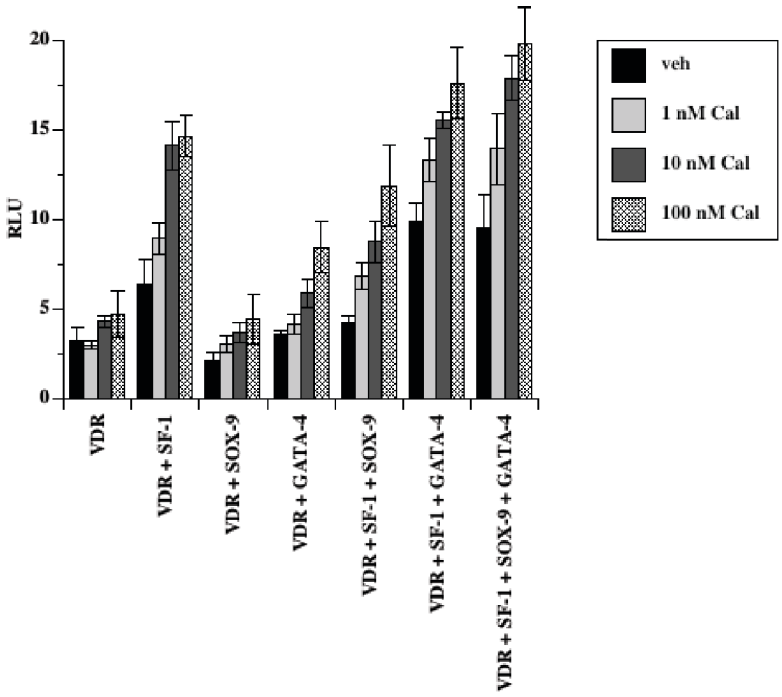

Fig. 2. Regulation of MIS promoter activity. HeLa cells were transfected with VDR, SF-1, GATA-4 and SOX9 expression vectors and the MIS promoter luciferase construct. Cells were then treated with graded concentrations of calcitriol and luciferase activity determined using the dual luciferase assay.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that MIS is produced by human prostate cancer cells
- Proved that MIS is a target of calcitriol
- Identified a VDRE in the MIS promoter
- Confirmed in vivo in cells that VDR acting via the VDRE up-regulates MIS
- Begun to study the enhancement of MIS anti-cancer activity when combined with calcitriol
- Submitted for publication our first paper showing calcitriol stimulation of MIS by a VDRE in the MIS promoter (now being revised, paper in the appendix)

REPORTABLE OUTCOMES

P.J. Malloy and D. Feldman. Mullerian Inhibitory Substance (MIS) is upregulated by 1,25-dihydroxyvitamin D_3 in LNCaP prostate cancer cells via a direct interaction of the vitamin D receptor with a vitamin D regulatory element in the MIS promoter.
Endocrine Society 88th Annual Meeting, Boston MA June 24-27, 2006.poster 3-55.

H.D. Mason, L. Hanna, S. Rice, H. Brain, P.J. Malloy, D. Feldman, M Brincat, R. Galea, S.A. Whitehead, and L.J. Pellat, Role of Anti-Mullerian Hormone (AMH) in anovulatory polycystic ovarian syndrome. Endocrine Society 88th Annual Meeting, Boston MA June 24-27, 2006. Oral presentation OR38-3.

P. J. Malloy, L. Peng and D. Feldman, Müllerian Inhibiting Substance (MIS) is up-regulated by calcitriol in LNCaP prostate cancer cells via a direct interaction of the vitamin D receptor with a vitamin D response element in the MIS promoter. DOD Prostate Cancer Meeting, Atlanta GA Sept 2007. poster

CONCLUSIONS

These studies add a new dimension to calcitriol action by adding the regulation of a new and previously unknown protein to the list of regulated substances by calcitriol. Since MIS is an important protein that regulates organ development and is currently in studies as an anti-cancer agent, this finding suggests that MIS may be useful in prostate cancer therapy and that calcitriol's regulation of MIS may contribute to its anti-cancer activity. Future studies will examine whether adding MIS to calcitriol will enhance the action of both molecules as anti-cancer agents. Our work has elucidated the mechanism by which calcitriol directly regulates the level of MIS expression by binding to a VDRE in the MIS promoter. Recent work has established the role of additional transcription factors in acting in concert with calcitriol to regulate MIS expression. Future work may include combinatorial studies using MIS plus calcitriol and a consideration of a new grant proposal to investigate calcitriol and MIS in ovarian cancer.

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Appendix

Manuscript submitted for publication. Currently in revision

Interaction of the vitamin D receptor with a vitamin D response element in the Müllerian Inhibiting Substance (MIS) promoter: regulation of MIS expression by calcitriol in prostate cancer cells

Running title: Calcitriol regulates MIS promoter activity

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Key words: calcitriol, 1,25-dihydroxyvitamin D, Mullerian inhibiting substance, anti-mullerian hormone, prostate, cancer, promoter

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Abbreviations: MIS, Mullerian inhibiting substance; VDR, vitamin D receptor, SF-1, steroidogenic factor 1; HVDRR, hereditary vitamin D resistant rickets, AMH, anti-mullerian hormone; VDRE, vitamin D response element; GMSA, gel mobility shift assay; MISRII, Mullerian inhibiting substance type II receptor.

ABSTRACT

Calcitriol (1,25-dihydroxyvitamin D_3) inhibits the growth of a variety of cancer cells including prostate cancer. Müllerian inhibiting substance (MIS) also exhibits anti-proliferative and pro-apoptotic actions on multiple cancer cells including prostate cancer. In this study, we investigated whether calcitriol regulated MIS expression in prostate cancer, an action that might contribute to its anti-proliferative activity. We identified a 15 bp sequence GGGTGAgcaGGGACA in the MIS promoter that was highly similar to DR3-type vitamin D response elements (VDREs). The MIS promoter containing the putative VDRE was cloned into a luciferase reporter vector. In HeLa cells transfected with the vitamin D receptor (VDR), MIS promoter activity was stimulated calcitriol. Co-expression of steroidogenic factor 1 (SF-1), a key regulator of MIS, increased basal MIS promoter activity that was further stimulated by calcitriol. Mutation or deletion of the VDRE reduced calcitriol-induced transactivation. In addition, the MIS VDRE conferred calcitriol responsiveness to a heterologous promoter. In gel shift assays, the VDR bound the MIS VDRE and the binding was increased by calcitriol. In chromatin immunoprecipitation assays, the VDR was bound to the MIS promoter and the binding was increased by calcitriol in LNCaP prostate cancer cells. Finally, we showed that MIS gene expression was upregulated by calcitriol in prostate cancer cells. In conclusion, we have demonstrated that MIS is a target of calcitriol action. MIS is upregulated by calcitriol via a functional VDRE that binds the VDR. Upregulation of MIS by calcitriol may be an important component of the anti-proliferative actions of calcitriol in some cancers.

The classical actions of calcitriol include the regulation of calcium and phosphate metabolism, actions that determine the quality of bone mineralization. These classical calcitriol actions prevent rickets in children and osteomalacia in adults as well as play a role in the prevention of osteoporosis (1). The biological actions of calcitriol are mediated by the VDR, a member of the steroid-thyroid-retinoid receptor superfamily of ligand activated transcription factors. Studies in VDR knockout mice (2, 3) and hereditary vitamin D resistant rickets (HVDRR) in humans (4) have revealed multiple biological consequences of VDR signaling. More recently, it has been recognized that calcitriol has a much wider range of actions that include pro-differentiation, anti-proliferation, pro-apoptosis, immuno-suppression and anti-inflammation (1, 5). These actions have led to potential uses of calcitriol and less calcemic calcitriol analogs in the treatment of diseases such as osteoporosis, cancer, immunologic diseases, diabetes, infection and psoriasis among others (6-16).

Mullerian-inhibiting substance (MIS, also known as Anti-Mullerian hormone or AMH) is a member of the transforming growth factor-β (TGFβ) superfamily that also includes activins, inhibins, and bone morphogenetic proteins (17). MIS is a glycoprotein that is secreted by Sertoli cells in testis and granulosa cells in the ovary. MIS binds to the MIS type II receptor (MISRII) a transmembrane serine threonine kinase and recruits the type I membrane receptor ALK2 in order to initiate downstream signaling (18-20). In developing male embryos, MIS initiates the regression of the Müllerian ducts that in a normal female embryo develop into the uterus, fallopian tubes, and upper vagina (21). Other roles for MIS have also been demonstrated. In Leydig cells MIS inhibits steroidogenesis (22, 23) and in the postnatal ovary MIS plays a role in follicle recruitment (24, 25).

Importantly, the growth of breast, cervical, endometrial, ovarian, and prostate cancer cells that express MISRII have been shown to be inhibited by MIS (26-38). In breast and prostate cancer cells, MIS up-regulates the immediate early gene 3 (*IER3*/*IEX-1S*) through an NF-κB-dependent mechanism (27, 29, 32). In breast cancer cells overexpression of *IER3* has been shown to inhibit cell growth (27). Furthermore, inhibition of prostate cancer cell growth by MIS was abolished by dominant negative IkB-α demonstrating that the growth inhibitory action of MIS is mediated by NF-κB in prostate (29).

In this report, we showed that the MIS promoter contains a functional vitamin D response element (VDRE) and its expression is regulated by calcitriol. Furthermore, we demonstrated that the MIS gene is upregulated by calcitriol in prostate cancer cells. Our findings demonstrate that MIS is a newly discovered direct target of the VDR that may have important implications in the anti-cancer activity of calcitriol.

Materials and Methods

Cell culture

HeLa cells were grown in DMEM containing 10% fetal bovine serum. LNCaP and PC-3 prostate cancer cells were grown in RPMI 1640 containing 5% fetal bovine serum. Cells were incubated at 37°C in 5% CO_2. Cells were obtained from the American Type Culture Collection (Manassas, VA).

Promoter Constructs

The MIS promoter sequence between –657 to +23 was amplified by PCR using genomic DNA and oligonucleotide primers designed with *Mlu I* restriction sites. The amplified product was cloned into the *Mlu I* site in the promoter-less luciferase reporter vector, pGL3-basic (Promega, Madison, WI). The sequence was verified by sequencing. A single point mutation in the putative VDRE sequence was constructed using the GenEditor site-directed mutagenesis kit (Promega). Deletion of the entire 15 bp VDRE sequence was performed by using a 40 base oligonucleotide primer that hybridized to 20 bases on either side of the 15 bp VDRE

sequence using the GenEditor kit. All clones were verified by sequencing. The heterologous MIS VDRE reporter was constructed by insertion of 4 copies of the MIS VDRE into the *Mlu* I site in pGL3-promoter luciferase reporter vector (Promega).

Gel Mobility Shift Assay (GMSA)

GMSA was used to analyze VDR binding to the putative VDRE in the MIS promoter using complementary oligonucleotides for the MIS VDRE. The osteopontin VDRE was run as a positive control. COS-7 cell extracts over-expressing the VDR were incubated with 10 nM calcitriol prior to the addition of the [^{32}P]-labeled MIS VDRE probe followed by electrophoresis on non-denaturing gels and autoradiography as previously described (39).

Transactivation Assays

The MIS promoter-luciferase plasmids were transfected into HeLa cells using HeLa Monster (Mirus Bio Corporation, Madison, WI). Cells were also co-transfected with pSG5-VDR and a control plasmid pRLnull to control for transfection efficiency. The transfected cells were treated with calcitriol for 24 hr. Luciferase activity was determined using the dual luciferase assay (Promega) and a Turner luminometer. In some experiments expression plasmids for SF-1 or SF-1 with a deletion of the LBD (SF-1ΔLBD) were co-transfected and the luciferase activity following measured calcitriol treatment. SF-1ΔLBD was constructed by digesting the SF-1 expression vector with *Sal* I followed by re-ligation as previously described (40).

Chromatin immunoprecipitation (ChIP) Assay

ChIP assays were used to demonstrate VDR binding to the MIS promoter in intact LNCaP cells as previously described (41). ChIP assays were performed using the ChIP kit from Upstate Biotechnology (Charlottesville, VA) with modifications (41). In brief, LNCaP cells were treated with 10 nM calcitriol for 2 hr and cross-linked by addition of 1% formaldehyde. The chromatin was sheared by sonication to obtain DNA fragments of an average of 200-1000 bp in size. An anti-VDR polyclonal antibody (H-81, Santa Cruz Biotechnology, Santa Cruz, CA) was added to the sheared DNA samples and incubated with constant rotation overnight at 4°C. Protein A agarose was added to capture the immune complexes. The agarose beads were washed, and the immunoprecipitates eluted with elution buffer. The cross-links were reversed by incubation at 65°C overnight in elution buffer containing 200 mM NaCl. The immunoprecipitated DNA fragments were purified using MiniElute Reaction Cleanup kits (Qiagen, Valencia, CA). PCR was then performed using primers flanking the MIS VDRE.

Real time RT-PCR

LNCaP cells were treated with 100 nM calcitriol for 6 hrs and total RNA isolated using RNAeasy spin columns (Qiagen). cDNA was prepared by reverse transcribing the RNA samples (5 µg) using Superscript III cDNA synthesis kit (Invitrogen, Carlsbad, CA). MIS gene expression was analyzed by real-time PCR using the Dynamo SYBR green qPCR kit (MJ Research, Reno, NV) and an Opticon 2 DNA engine (MJ Research). Primers were designed to span intron-exon junctions and to amplify a 200-300 bp product. Melting curves were analyzed to demonstrate the purity of the PCR products. The TATA box-binding protein gene was used as a control for cDNA input (42).

Statistical analyses

Transient transfections were performed in triplicate, and each experiment was repeated at least three times. The data were analyzed by the Student's *t* test and significant differences were designated as $P < 0.05$.

Results

The human MIS promoter is contained within a 789 bp sequence between the end of the SF3A2 gene and the start of the MIS coding sequence (43) (Fig. 1A). To determine whether putative VDREs were present in the MIS promoter we used an *in silico*-based method to scan the promoter region of the MIS gene. We first analyzed the entire MIS promoter for VDREs using the Genetics Computer Group MAP program and a transcription factor database. The program identified a single VDRE-like sequence in the MIS promoter. As

shown in Fig. 1B, the putative VDRE was located at -395 to –381 relative to the MIS translational start site. The putative VDRE sequence was located upstream of known transcription factor binding sites for SF-1, SOX-9 and GATA-4 (Fig. 1B). The MIS VDRE exhibits a direct repeat 3 (DR-3) motif containing two hexameric sequences separated by a 3 bp spacer that is highly similar to several previously characterized VDREs. As shown in Table 1, the MIS VDRE is highly homologous to both the human and rat osteocalcin VDREs. The MIS VDRE (GGGTGAgcaGGGACA) and the human osteocalcin VDRE (GGGTGAacgGGGGCA) differ by only one nucleotide base in the 3-prime hexamer (Table 1).

Having identified a putative VDRE in the MIS promoter, we amplified a 680 bp DNA fragment from –657 to +23 and cloned it into the pGL3-basic luciferase reporter plasmid (Fig. 1C). We then transfected HeLa cells with the WT VDR cDNA expression vector and the MIS promoter reporter plasmid and examined transactivation following treatment with calcitriol. As shown in Fig. 1D, in HeLa cells transiently transfected with the WT VDR, calcitriol treatment induced a dose-dependent increase in luciferase activity. At 100 nM calcitriol there was an approximately 1.8-fold increase in luciferase activity versus vehicle treated cells. In cells transfected with the pSG5 vector control, calcitriol treatment failed to stimulate luciferase activity. These results demonstrated that the MIS promoter responds to calcitriol treatment.

Since SF-1 is a known major regulator of MIS promoter activity (40), we next determined the effects of co-expression of SF-1 and VDR on MIS promoter activity. As shown in Fig. 2, in the absence of calcitriol the basal MIS promoter activity was increased approximately 2.5-fold by co-expression of SF-1 and VDR compared to expression of VDR alone. In the presence of SF-1 and VDR calcitriol caused a dose-dependent increase in MIS promoter activity. At 100 nM calcitriol there was an approximately 2.4-fold increase in MIS promoter activity compared to vehicle control. These results demonstrated that VDR and SF-1 cooperate to stimulate MIS promoter activity and that calcitriol is essential for maximum activity. The slight increase in MIS promoter activity at the 100 nM calcitriol concentration in the control transfected with the pSG5 vector and SF-1 was most likely due to the presence of the small amount of endogenous VDR in the COS-7 cells.

To confirm that the induction of the MIS promoter by calcitriol was mediated via the putative VDRE sequence, we constructed two mutations in the MIS VDRE (Fig. 3A). In one mutant, the 3-prime hexamer sequence GGGACA was mutated to G**T**GACA (MISmut1). In the second mutant, the entire 15 base GGGTGAgcaGGGACA VDRE sequence was deleted (MISΔVDRE). As shown in Fig. 3B, in HeLa cells co-transfected with VDR and SF-1, calcitriol induced a dose-dependent increase in WT MIS promoter activity. On the other hand, the single point mutation significantly reduced calcitriol-induced transactivation when compared to WT MIS promoter. Furthermore, deletion of the MIS VDRE sequence abolished transactivation by calcitriol. These results demonstrated that the VDR stimulated MIS promoter activity through the MIS VDRE sequence.

To further demonstrate that the VDRE sequence specifically responds to calcitriol, the 15 bp VDRE sequence was cloned into pGL3-promoter, a heterologous promoter luciferase reporter vector containing an SV40 promoter (Fig. 4A). As shown in Fig. 4B, when HeLa cells transfected with the MIS VDRE-pGL3-promoter construct were treated with calcitriol, a dose-dependent increase in transactivation activity up to 2-fold was observed. HeLa cells transfected with the pGL3-promoter vector without an insert exhibited no calcitriol-induced increase in transactivation (data not shown). These results demonstrated that the 15 bp MIS VDRE sequence conferred calcitriol responsiveness to a heterologous promoter.

We next determined whether the VDR binds directly to the MIS VDRE in vitro using gel shift assays. For these assays we used COS-7 cells that express endogenous RXR but only a small amount of VDR. As shown in Fig. 5A, when extracts from COS-7 cells transfected with the pSG5 vector alone were incubated with radio-labeled oligonucleotide probes, no band shifts were detected with either the MIS VDRE

or the well-characterized osteopontin VDRE control. A second control was the VDR E420K a mutant defective in coactivator binding but not ligand binding, RXR heterodimerization or DNA binding (39). When extracts of COS-7 cells that were transfected with the VDR WT or VDR E420K cDNA expression vectors in the absence of calcitriol, band shifts were detected with both VDRE sequences (Fig. 5A). Addition of calcitriol further increased the band intensities demonstrating that the VDR was bound to the VDRE sequences.

Having shown that MIS is a calcitriol target gene, we next examined whether the VDR interacted with the MIS-VDRE *in vivo* using ChIP assays. LNCaP cells were treated with vehicle or 10 nM calcitriol for 2 hr prior to ChIP assay. As shown in Fig. 5B, the sequence containing the MIS-VDRE was immunoprecipitated by the VDR antibody but not by the non-specific IgG control antibody. In the absence of calcitriol VDR binding to the MIS VDRE was observed. In the presence of calcitriol VDR binding to the MIS VDRE was increased. These results demonstrated that the VDR interacts with the MIS-VDRE *in vivo*.

Both calcitriol and MIS have been shown to inhibit the growth of many different types of cancer cells including prostate cancer cells. Since our lab has been investigating the role of calcitriol in regulating prostate cancer cell growth, we were interested in determining whether calcitriol regulated MIS expression in prostate cancer cells as an additional mechanism for the anti-proliferative affects of calcitriol. To examine this hypothesis, we treated LNCaP and PC-3 human prostate cancer cells and the human primary prostate cancer cell strain JBEpz with calcitriol and examined MIS gene expression by real-time RT-PCR. As shown in Fig. 6, calcitriol induced an approximately 2-3 fold increase in MIS gene expression in both LNCaP and PC-3 cells. Lower but significant induction was also observed in the JBEpz cells.

Discussion

We have demonstrated that the MIS promoter contains a functional VDRE and that calcitriol upregulates MIS gene expression via this response element. The MIS VDRE is highly similar to the human and rat osteocalcin VDREs, classical vitamin D target genes (Table 1). Co-expression of VDR and SF-1 increased basal MIS promoter activity that was further stimulated by calcitriol. Mutagenesis or deletion of the MIS VDRE significantly reduced or abolished responsiveness to calcitriol. In gel shift assays, the VDR was bound to the MIS VDRE and the binding was increased by calcitriol. The 15 bp VDRE also conferred calcitriol responsiveness to a heterologous promoter. In vivo we showed that the VDR was present on the MIS VDRE using ChIP assays and that calcitriol induced MIS gene expression in prostate cancer cells. These data demonstrate that the MIS promoter contains a functional VDRE that binds the VDR and is responsive to calcitriol.

Although MIS is most known for its activity to initiate regression of Mullerian structures during male fetal development (20), other post-natal actions have been documented. MIS exhibits important actions on steroidogenesis (22, 23), follicle development (24, 25, 44, 45), ovarian and testicular function (46) and has been linked to polycystic ovarian disease (PCOS) (47-49). Whether calcitriol and VDR contribute to these activities by induction of MIS is currently unknown and warrants further investigation.

It has been suggested that the MIS locus is in the open chromatin state since a significant number of spliceosome associated protein 62 (SAP62) transcripts continue through the MIS gene (43). In our ChIP assays, we demonstrated that in the absence of calcitriol the VDR is associated with the MIS-VDRE *in vivo*. The presence of the VDR on the MIS promoter in the absence of calcitriol also indicates that the MIS locus is in the open state. MIS exhibits precise regulation despite its apparent open chromatin state indicating that its expression is under stringent control. Since the VDR has been shown to interact with corepressors and silence gene activity (50, 51), it raises the possibility that the unliganded VDR silences MIS gene expression in the absence of calcitriol. Dax-1 has also been shown to inhibit MIS expression by interacting with SF-1 (52).

Our initial interest to study MIS in relation to vitamin D stems from our investigation of various pathways to inhibit prostate cancer

development or progression (5). We are especially focused on the potential of using calcitriol in combination therapy with other anti-cancer drugs (5, 53-55). However, another reason for our interest in MIS was the finding in one of the cases of hereditary vitamin D resistant rickets (HVDRR) that we previously reported (56, 57). This child, who has since died, had two rare genetic disorders in addition to HVDRR, generalized congenital lipodystrophy of the Berardinelli-Seip type (BSCL), and persistent Mullerian duct syndrome (PMDS). We found that the basis of his HVDRR was a mutation in the VDR LBD (H305Q) that altered the contact point for the 25-hydroxyl group in calcitriol (57). BSCL, a rare autosomal recessive disorder, was found to be caused by a splice site mutation in his BSCL2 gene (58). The BSCL2 gene product, seipin, is a transmembrane protein of unknown function localized in the endoplasmic reticulum (59). PMDS is usually caused by mutations in the MIS gene or the MIS receptor (MISRII) gene and is characterized by the presence of Mullerian derivatives in males (60-64). However, since the child already had two proven rare and unrelated autosomal mutations, we wondered whether this child could possibly harbor three unique rare mutations, which on a statistical basis would be extremely remote. Alternatively, we speculated that his PMDS might be caused by a downstream defect due to the mutated VDR. Our finding and that MIS is regulated in part by calcitriol and the fact that VDR is expressed in Sertoli cells (65-70) makes this hypothesis feasible. The molecular basis for PMDS in the patient had not been discovered. PMDS has not been described in other cases of HVDRR. However, the presence of retained Mullerian ducts may not cause symptoms in boys at an early age, and the presence of PMDS in other HVDRR boys may have been missed. In any case, the current study does prove that MIS is directly regulated by calcitriol and the loss of this action due to the mutation in the VDR may have caused PMDS in this child. We hope that any future cases of boys with HVDRR will be carefully checked for PMDS.

In females MIS is expressed in granulosa cells of the ovary (71, 72). Interestingly, in one VDR knockout mouse model uterine hypoplasia with impaired folliculogenesis was found in female reproductive organs but not vitamin D-deficient animals (3). One explanation for these defects was that there was impaired estrogen synthesis in the knockout mice. However, our finding that the MIS gene is regulated by the VDR indicates a possible role for VDR in female reproduction. In addition, PCOS has also been associated with high levels of MIS (47-49). The role of calcitriol and/or the VDR in the overproduction of MIS in PCOS warrants further investigation.

More recently, it has become clear that MIS has actions to inhibit cancer growth and MIS is currently under intense investigation for use as an anti-cancer drug (35, 73, 74). Data indicate that MIS has activity against a variety of cancers including uterine, cervical, ovarian, breast and prostate cancer to name a few (26-35, 37, 38, 75). In prostate cancer, MIS exerts dual actions to both inhibit androgen synthesis while also promoting tumor regression (22, 29, 76). Calcitriol also has multiple actions to prevent and inhibit prostate cancer growth. These actions include cell cycle arrest, pro-differentiation, apoptosis, anti-angiogenesis, inhibition of invasion and metastasis, and anti-inflammatory activity (5, 6, 13, 16, 77-80). The findings presented here demonstrate that upregulating MIS is another anti-cancer action in the prostate. What proportion of the anti-proliferative actions of calcitriol in prostate are due to stimulation of MIS is not yet clear. However, stimulation of endogenous MIS production by calcitriol would be additive to the anti-cancer activity of exogenous MIS used as cancer therapy. This leads us to speculate that combination therapy with MIS plus calcitriol should be more efficacious than MIS alone and should be considered in the investigation of MIS utility as an anti-cancer agent.

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TABLES

Table 1. Comparison of the human MIS VDRE and several previously described VDREs

Gene	Location	Sequence
Human MIS	-395/-381	GGGTGA gca GGGACA
Human Osteocalcin	-499/-485	GGGTGA acg GGGGCA
Rat Osteocalcin	-460/-446	GGGTGA atg AGGACA
Human CYP24A1	-169/-155 (proximal)	AGGTGA gcg AGGGCG
Human CYP24A1	-291/-277 (distal)	AGTTCA ccg GGTGTG
Human IGFBP-3	-3296/-3282	GGTTCA ccg GGTGCA

FIGURE LEGENDS

Figure 1. The human MIS promoter containing a putative VDRE is activated by calcitriol. A, The human MIS gene on chromosome 19 is located between the SF3A2 gene and the JSRP1 gene. The MIS transcriptional start site is located only 748 bp downstream of the termination codon of the SF3A2 gene. Arrows indicate direction of transcription. B, Using *in silico* analysis, we identified a putative VDRE in the MIS promoter. The VDRE is located at nucleotides –381 to –396 relative to the ATG translation start site. The location of transcription factor binding sites for SF-1, SOX9 and GATA-4 that regulate MIS promoter activity are also shown. C, A 680 bp fragment (–657 to +23) of the MIS promoter was cloned into the promoter-less luciferase reporter vector pGL3-basic to generate the MIS promoter reporter construct (MISpro). D, Transactivation assays in HeLa cells transfected with the pSG5 vector without an insert or pSG5-VDR expression vector and the MIS promoter luciferase reporter construct. Cells were treated with vehicle or calcitriol (Cal) for 24 hr. Luciferase activity was measured using the dual luciferase assay. Shown is a representative experiment of at least three independent experiments. Values represent mean ±SD of triplicate transfections. *, calcitriol treatment significantly different from the vehicle-treated control by Student's *t* test. n.d., not significantly different.

Figure 2. Steroidogenic factor 1 (SF-1) and VDR cooperate to increase MIS promoter activity in response to calcitriol. A, MIS promoter-luciferase construct. B, Transactivation assays in HeLa cells transfected with pSG5 alone, VDR alone, or the combination of VDR and SF-1 expression vectors and the MIS promoter-luciferase construct. Cells were treated with vehicle or calcitriol (Cal) for 24 hr and luciferase activity measured. Shown is a representative experiment of at least three independent experiments. Values represent mean ±SD of triplicate transfections. *, calcitriol treatment significantly different from the vehicle-treated control by Student's *t* test.

Figure 3. Mutations in the MIS VDRE reduce calcitriol responsiveness. A, A single G to T point mutation in the 3-prime hexamer of the VDRE (MISpro mut-1) and a 15 bp deletion of the entire VDRE (MISpro ΔVDRE) were created in the MIS promoter-luciferase construct (MISpro). B, Transactivation assays in HeLa cells transfected with VDR and SF-1 expression vectors and the MIS promoter luciferase constructs. Cells were treated with vehicle or calcitriol (cal) for 24 hr and luciferase activity measured. Shown is a representative experiment of at least three independent experiments. Values represent mean ±SD of triplicate transfections. *, calcitriol treatment significantly different from the vehicle-treated control by Student's *t* test.

Figure 4. The MIS VDRE confers calcitriol responsiveness to a heterologous promoter. A, The 15 bp MIS VDRE was cloned upstream of the SV40 promoter in the reporter vector pGL3-promoter (pGL3pro). B, Transactivation assays in HeLa cells transfected with a VDR expression vector and the MIS VDRE-pGL3pro luciferase reporter construct. Cells were treated with vehicle or calcitriol for 24 hr and luciferase activity measured. Shown is a representative experiment of at least three independent experiments. Values represent mean ±SD of triplicate transfections. *, calcitriol treatment significantly different from the vehicle-treated control by Student's *t* test.

Figure 5. The VDR binds to the MIS VDRE *in vitro and in vivo*. A, [^{32}P]-labeled MIS and osteopontin (OP) VDREs were incubated with COS-7 extracts transfected with vector alone (pSG5), WT VDR, or VDR E420K a coactivator binding defective mutant. Samples were incubated with and without 10 nM calcitriol for 30 min at ambient temperature. The samples were then electrophoresed on 5% polyacrylamide gels in 0.5X Tris-borate buffer for 170 min at 70 volts. Bands were visualized by autoradiography. B, LNCaP cells were treated with and without 10 nM calcitriol for 2 hr at 37°C. The cells were then fixed with 1% formaldehyde for 10 min at ambient temperature. The samples were washed with phosphate-buffered saline and then analyzed by chromatin immunoprecipitation (ChiP)

assays. ChiP assays were performed with VDR and control IgG antibodies. Input represents DNA extracted prior to ChiP assay. Cal, calcitriol.

Figure 6. Calcitriol up-regulates MIS in prostate cancer cells. LNCaP and PC-3 prostate cancer cell lines and JBEpz, a primary prostate cancer isolated from the peripheral zone were treated with vehicle (open bar) or 100 nM calcitriol (hatched bar) for 6 hr. RNA was isolated and MIS gene expression analyzed by real time RT-PCR. Values were normalized to TBP expression and represent mean ±SD of triplicate assays.

Figure 1

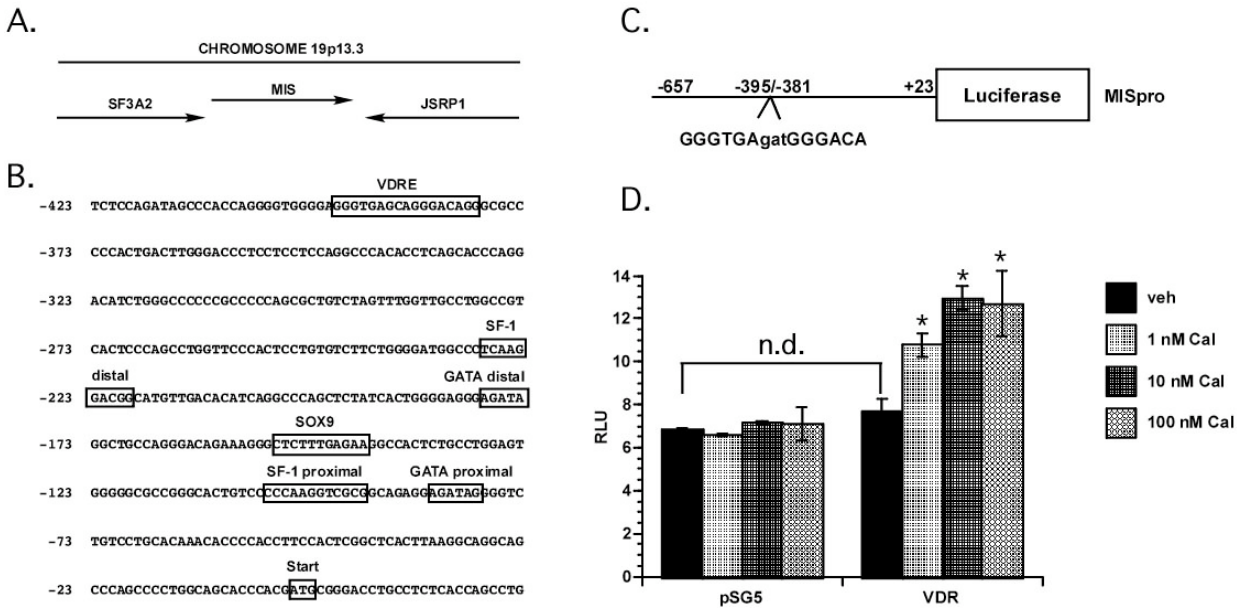
A.
CHROMOSOME 19p13.3
SF3A2
MIS
JSRP1
B.
VDRE
-423 TCTCCAGATAGCCCACCAGGGGTGGGGAGGGTGAGCAGGGACAGGGCGCC
-373 CCCACTGACTTGGGACCCTCCTCCTCCAGGCCCACACCTCAGCACCCAGG
-323 ACATCTGGGCCCCCGCCCCCAGCGCTGTCTAGTTTGGTTGCCTGGCCGT
SF-1
-273 CACTCCCAGCCTGGTTCCCACTCCTGTGTCTTCTGGGGATGGCCCTCAAG
distal
GATA distal
-223 GACGGCATGTTGACACATCAGGCCCAGCTCTATCACTGGGGAGGGAGATA
SOX9
-173 GGCTGCCAGGGACAGAAAGGGCTCTTTGAGAAGGCCACTCTGCCTGGAGT
SF-1 proximal
GATA proximal
-123 GGGGGCGCCGGGCACTGTCCCCCAAGGTCGCGGCAGAGGAGATAGGGGTC
-73 TGTCCTGCACAAACACCCCACCTTCCACTCGGCTCACTTAAGGCAGGCAG
Start
-23 CCCAGCCCCTGGCAGCACCCACGATGCGGGACCTGCCTCTCACCAGCCTG
C.
-657
-395/-381
+23
Luciferase
MISpro
GGGTGAgatGGGACA
D.
RLU
n.d.
pSG5
VDR
veh
1 nM Cal
10 nM Cal
100 nM Cal

Figure 2

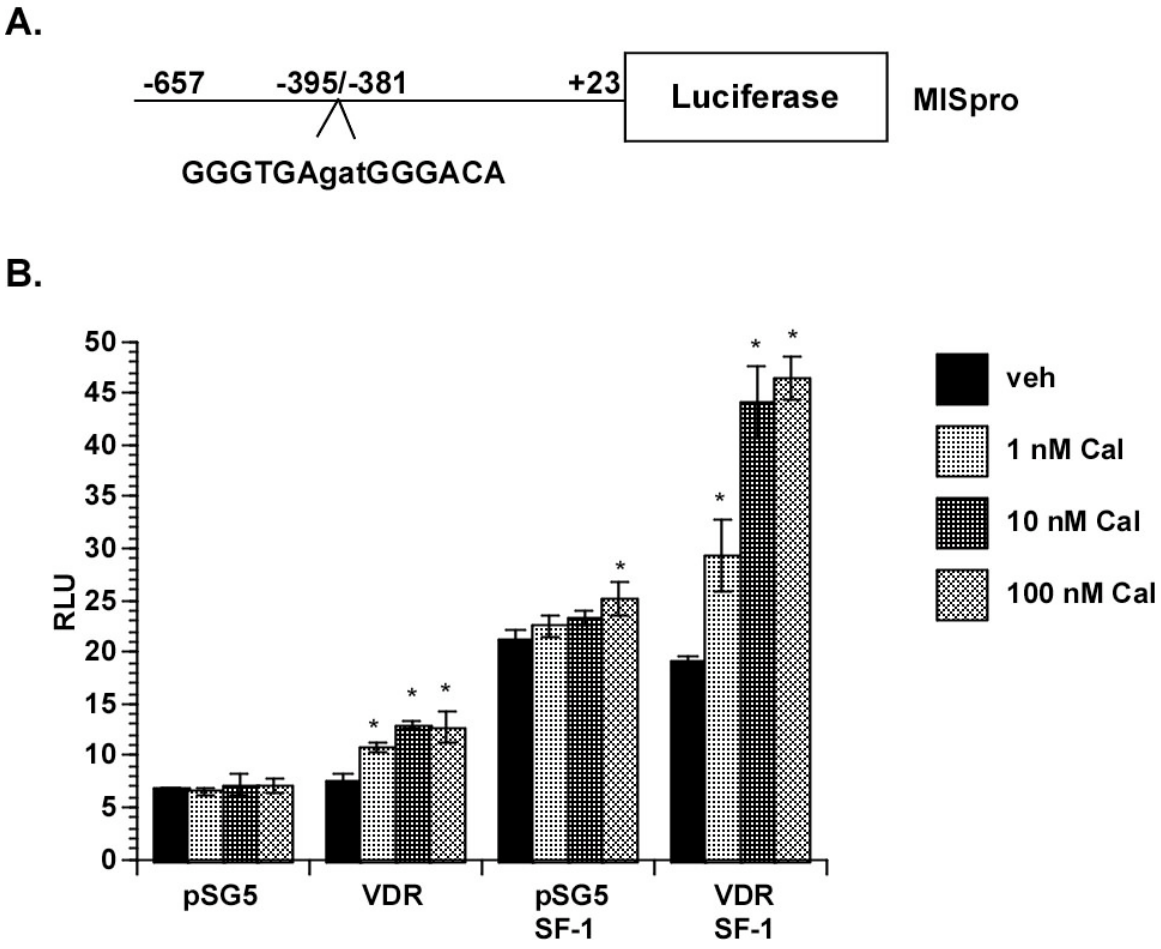
A.
-657
-395/-381
+23
Luciferase
MISpro
GGGTGAgatGGGACA
B.
RLU
50
45
40
35
30
25
20
15
10
5
0
pSG5
VDR
pSG5
SF-1
VDR
SF-1
veh
1 nM Cal
10 nM Cal
100 nM Cal

Figure 3

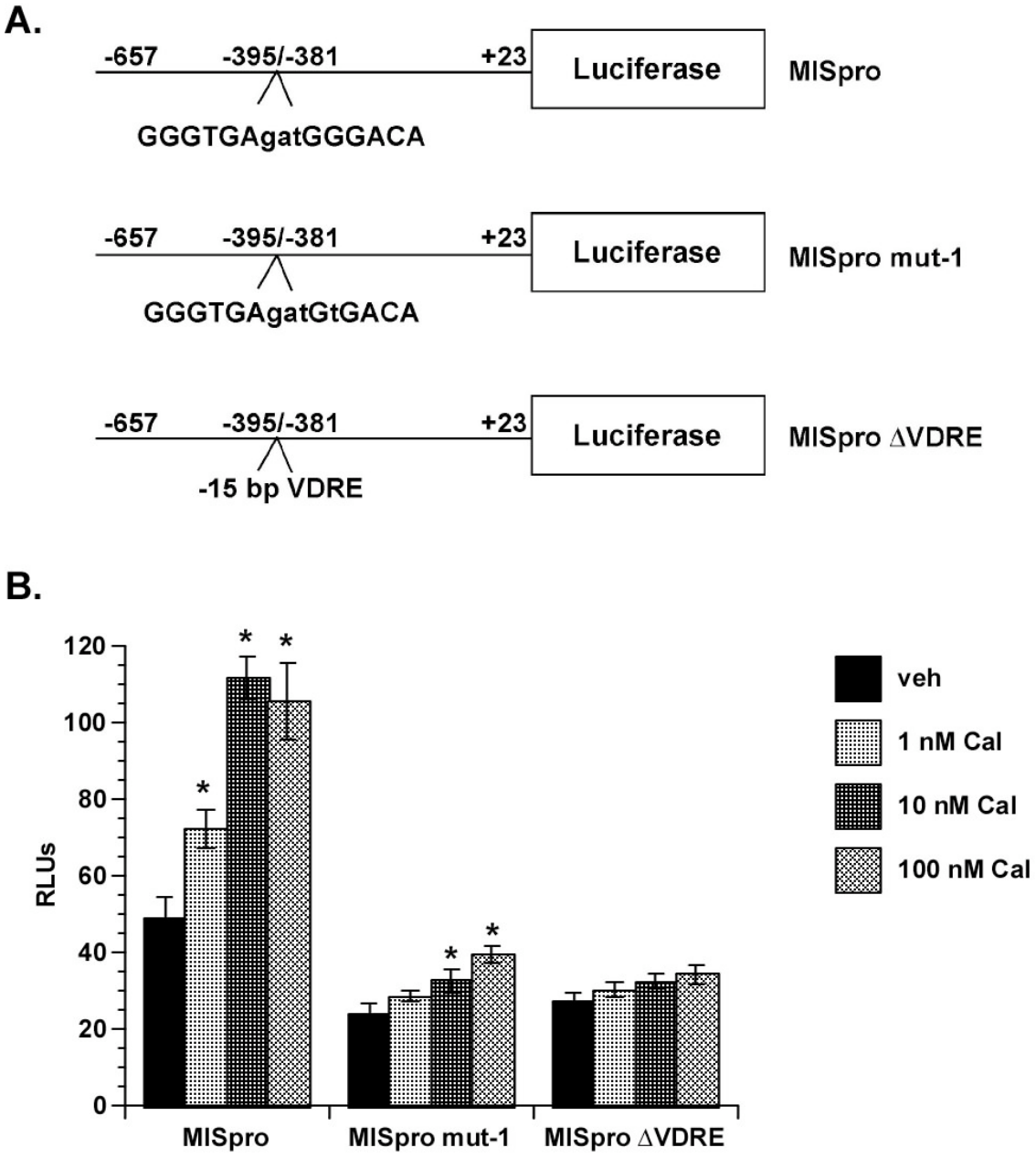
A.
-657
-395/-381
+23
Luciferase
MISpro
GGGTGAgatGGGACA
-657
-395/-381
+23
Luciferase
MISpro mut-1
GGGTGAgatGtGACA
-657
-395/-381
+23
Luciferase
MISpro ΔVDRE
-15 bp VDRE
B.
RLUs
120
100
80
60
40
20
0
*
*
*
*
MISpro
MISpro mut-1
MISpro ΔVDRE
veh
1 nM Cal
10 nM Cal
100 nM Cal

Figure 4

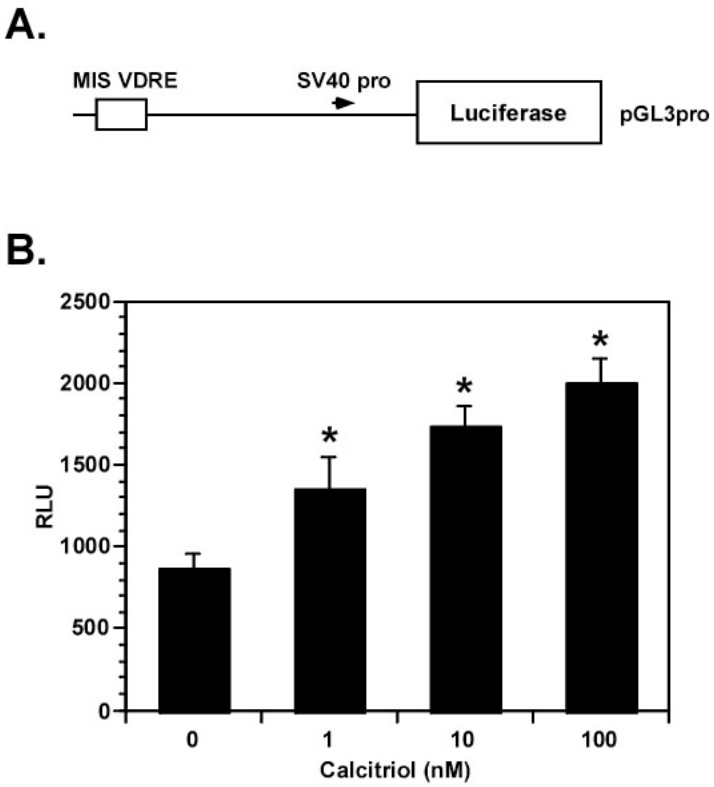
A.
MIS VDRE
SV40 pro
Luciferase
pGL3pro
B.
2500
2000
1500
1000
500
0
RLU
*
*
*
0
1
10
100
Calcitriol (nM)

Figure 5

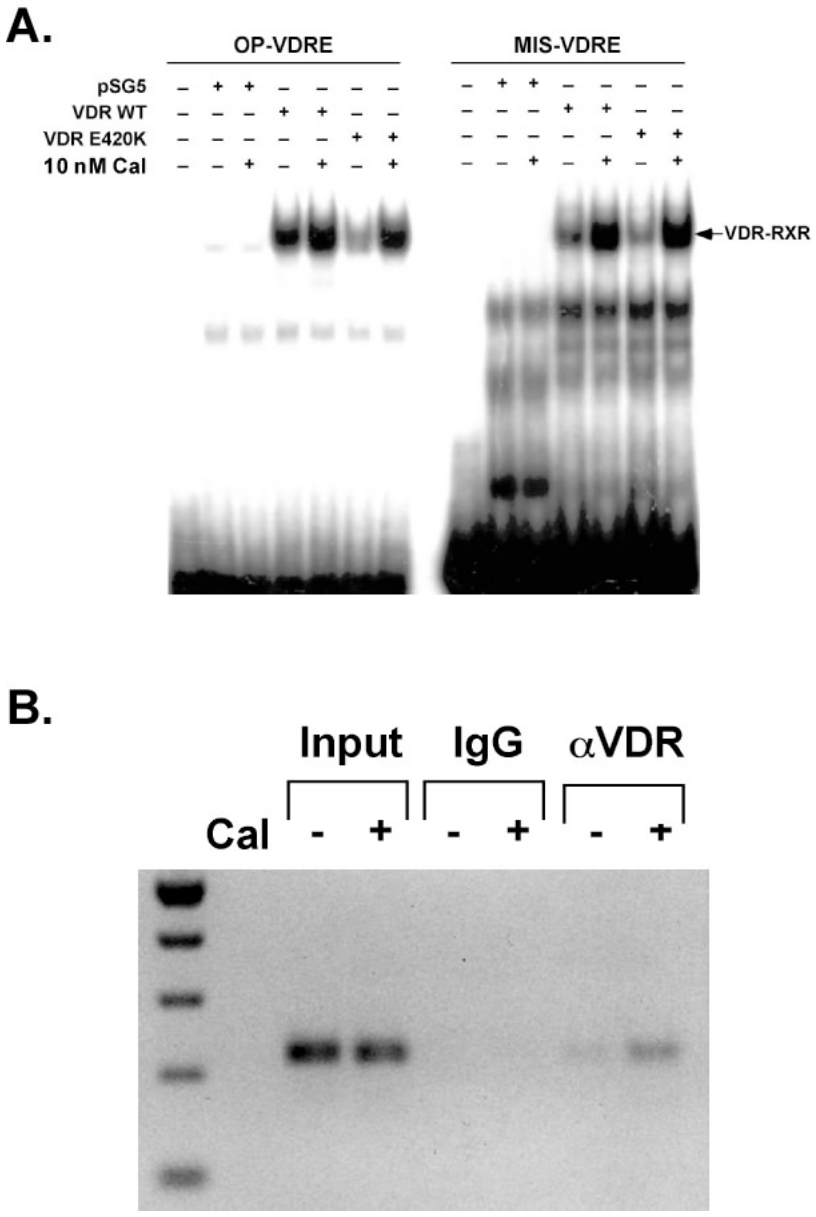
A.
OP-VDRE
MIS-VDRE
pSG5
VDR WT
VDR E420K
10 nM Cal
VDR-RXR
B.
Input
IgG
αVDR
Cal
- + - + - +

Figure 6